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HEME OXYGENASE ACTIVITY, DRUG METABOLISM, AND ASCORBIC ACID DISTRIBUTION IN THE LIVERS OF ASCORBIC ACID-DEFICIENT GUINEA PIGS*,†

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Abstract—The influence of ascorbic acid (AA) on microsomal heme oxygenase (MHO) (EC 1.14.99.3), drug metabolism and AA distribution was studied in livers and liver cytosols isolated from guinea pigs. Aminopyrine *N*-demethylase and cytochrome P-450 content were examined in guinea pigs on days 0, 6, 12 and 19 after being placed on a basal diet deficient in AA. Plasma AA seems to reflect total liver AA; however, AA from the liver cytosol (100,000 *g* soluble fraction) component decreased at a slower rate than total liver AA as deficiency progressed. The decrease in hepatic aminopyrine *N*-demethylase and in cytochrome P-450 content was related to the decrease in cytosolic AA. Perfusion of livers from guinea pigs not depleted of AA resulted in a 71 per cent loss of extracellular AA. Liver perfusions also resulted in 26.2 and 16.0 per cent decreases in cytochrome P-450 content from AA-deficient and AA-supplemented guinea-pigs (25 mg/100 g) respectively. These data suggest that a labile soluble pool of AA may have some influence on cytochrome P-450 content and drug metabolism. MHO activity was decreased significantly ($P < 0.05$) in the livers from AA-deficient guinea pigs compared to AA-supplemented guinea pigs. In a separate experiment, guinea pigs were given either the basal diet alone or the basal diet and either 1 or 50 mg AA/100 g for 28 days. MHO activity was found to increase significantly with increasing doses of AA ($P < 0.005$). These results suggest a dose-related dependence of MHO on AA and that AA deficiency does not produce an increase in hepatic heme catabolism via increased MHO activity.

The primary known nutritional role of ascorbic acid (AA) is in the prevention and cure of scurvy. Other roles have been suggested for AA, including the prevention of the common cold. A role that has been suggested and has been supported, at least in the guinea pig, is the role of AA in hepatic [1–3] and extrahepatic [4] drug or toxicant metabolism. The significance of such a role is quite important from a therapeutic standpoint; however, caution must be emphasized in the extrapolation of results from studies in guinea pigs to values for primates [5], including man [6].

Despite 35 years of extensive research, the mechanism of the influence AA has on drug metabolism remains unknown. The labile nature of AA and the lack of highly sensitive and specific methods of AA analysis have contributed to the failure of determining whether or not the decrease of drug metabolism is due directly to a decrease of microsomal AA. Because of the complexity of the hepatic microsomal electron transport system, there are a number of possible areas in which AA could participate. Areas investigated in AA deficiency which resulted in no correlation included: increased breakdown due to lipid peroxidation [7], qualitative changes in microsomal lipids [7], and changes in heme precursors for cytochrome P-450 [8].

In this study, we were concerned whether or not the decreases in drug metabolism that occur in AA-deficient guinea pigs are related to increased degradation of cytochrome P-450 by the induction of the rate-limiting enzyme MHO (EC 1.14.99.3). In addition, we attempted to determine indirectly whether or not the loss of cytochrome P-450 is a function of decreased microsomal AA.

MATERIALS AND METHODS

Animals and diets. In the following two dietary experiments, young male Hartley guinea pigs (Charles River, Wilmington, MA) with an initial weight of 200–250 g were maintained on specific diets and water *ad lib.* Animals were housed in stainless steel wire-bottomed cages equipped with an automatic watering system. The animals were housed one to a cage for the entire length of the experiments. The basal diet was a pelleted AA-free diet made according to the specifications developed by Reid and Briggs [9]. The AA content of the basal diet was less than 0.02 mg/g of diet.

In the first dietary experiment, fifty guinea pigs were fed the basal AA-free diet of which sixteen animals were dosed (p.o.) daily, once a day, with an aqueous solution of 25 mg AA/100 g of body weight. This aqueous solution of AA was made fresh daily and was administered via the mouth by micropipet. The dosage volume was 100 μl/100 g of body weight. Animals fed the AA-free diet were killed in groups of four on days 0, 6, 12 and 19 after starting the dietary regimen. Food was withheld from the animals 16 hr prior to being decapitated on each of the specified days. Blood was collected into tubes containing EDTA. Livers were isolated-

* The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

† In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" as promulgated by the Committee on Revision of the Guide for Laboratory Animal Resources, National Research Council.

excised, cleaned of nonhepatic tissues, weighed, and prepared for biochemical analysis. The eighteen remaining guinea pigs fed the AA-free basal diet alone and the sixteen guinea pigs fed the basal diet supplemented with AA were fasted for 16 hr and were decapitated 25 days after starting the first dietary regimen. Blood was collected into tubes containing EDTA. The livers from half of each group were perfused *in situ*. Livers were isolated-excised, cleaned of nonhepatic tissues, weighed, and prepared for biochemical analysis.

In the second dietary experiment, twenty-one guinea pigs were fed the AA-free basal diet for 25 days. The animals were divided into three groups of seven guinea pigs per group. The groups of animals were dosed daily, p.o., with 0, 1 or 50 mg AA/100 g of body weight, representing a deficient group, a group dosed with twice the level recommended by the National Research Council [10], and a group with a plasma saturating level of AA respectively. Food was withheld from the animals 16 hr prior to being decapitated at day 25 after starting the dietary regimen. Blood was collected and livers were excised; the blood and excised livers were then prepared for biochemical analysis.

Tissue preparation. Whole blood was centrifuged at 1400 *g* for 20 min at 5°; the clear plasma was stabilized with 10% trichloroacetic acid (TCA) and recentrifuged. The acid extract was analyzed for AA. When liver perfusion was necessary, the livers were perfused *in situ* with 0.15 M KCl, excised, weighed and minced cold. Minced perfused livers were homogenized with 2 vol. of cold 0.05 M Na^+/K^+ phosphate buffer, pH 7.4, in 0.15 M KCl. Homogenization was done in a glass and Teflon homogenizer by three strokes of the pestle. Minced nonperfused livers were homogenized with 4 vol. of the same cold buffer. Tissue subcellular fractions were prepared as follows. Homogenates were centrifuged at 750 *g* for 15 min to remove nuclear and cellular debris. The resultant supernatant fraction was removed and recentrifuged for 20 min at 12,000 *g*. Portions of the 12,000 *g* post-supernatant fraction were centrifuged at 100,000 *g* for 60 min in a Beckman ultracentrifuge. The resultant supernatant fraction (cytosol) was removed and AA was determined. The microsomal pellet was resuspended in a small volume of buffer for cytochrome P-450 determination. To remove residual hemoglobin from microsomes isolated from nonperfused livers, resuspended (washed) microsomes were recentrifuged at 100,000 *g* for 30 min. The 100,000 *g* post-supernatant fraction was discarded and the microsomal pellet was resuspended again in a small volume of buffer for cytochrome P-450 determination.

When it was necessary to assay for MHO activity, portions of the 750 *g* post-supernatant fraction taken from homogenates of perfused livers were recentrifuged at 18,000 *g* for 10 min. Livers were perfused to remove blood heme compounds that would interfere with MHO assay. MHO activity was determined in the 18,000 *g* post-supernatant fraction.

Biochemical analysis. Plasma, minced portions of whole liver and liver cytosols were stabilized in 10% TCA. The stabilized minced portions of whole livers were homogenized directly in glass and Teflon homogenizers. Ascorbic acid was determined by a procedure similar to that developed by Roe [11] modified for tissue [12]. In this modification, the addition of thiourea and a lower incubation temperature (37°) prevent interference from non-AA chromogen. The sensitivity of the modified AA assay is 1–5 μg/ml.

Microsomal cytochrome P-450 content was estimated on 2–3 mg protein/ml of resuspended microsomal pellet by the dithionite difference spectrum [13] by using a Beckman DK 2A split-beam recording spectrophotometer. The Nash procedure for formaldehyde [14] was used to determine aminopyrine *N*-demethylase activity in the 12,000 *g* post-supernatant fraction [15]. The production of formaldehyde was linear for at least 60 min.

MHO activity was determined in the 18,000 *g* post-supernatant fraction by the procedure of Correia and Schmid [16]. Bilirubin, formed in the presence of excess biliverdin reductase, was measured by the difference spectrum (469 nm) with the use of an Aminco–Chance DW-2 dual wavelength/split-beam recording spectrophotometer.

Protein was determined by the method of Miller [17].

Statistics. Data were analyzed statistically by Student's *t*-test or by using a distribution-free test for ordered alternatives [18]. Differences between values of group means were considered significant only if the analysis of variance indicated a probability of less than 0.05.

RESULTS

We found that hepatic aminopyrine *N*-demethylase activity decreased to 76, 52 and 32 per cent of normal

Table 1. Effect of AA depletion on liver weights, hepatic aminopyrine demethylase activity and hepatic microsomal cytochrome P-450*

Days on AA-free regimen	Liver wt (g)	Aminopyrine *N*-demethylase†	Cytochrome P-450‡
0 (normal)	9.86 ± 0.57	21 ± 1.0	1.33 ± 0.086
6	8.52 ± 0.44	16 ± 0.7§	1.15 ± 0.044
12	9.98 ± 0.38	11 ± 3.2§	0.87 ± 0.225
19	10.41 ± 0.79	7 ± 1.1§	0.63 ± 0.083§

* N = four animals per group. Values are expressed as means ± S.E.M. Aminopyrine *N*-demethylase activity and cytochrome P-450 content were determined on non-perfused livers.

† Expressed in nmoles/hr/mg of microsomal protein.

‡ Expressed in nmoles/mg of microsomal protein.

§ Significantly different from normal ($P < 0.05$).

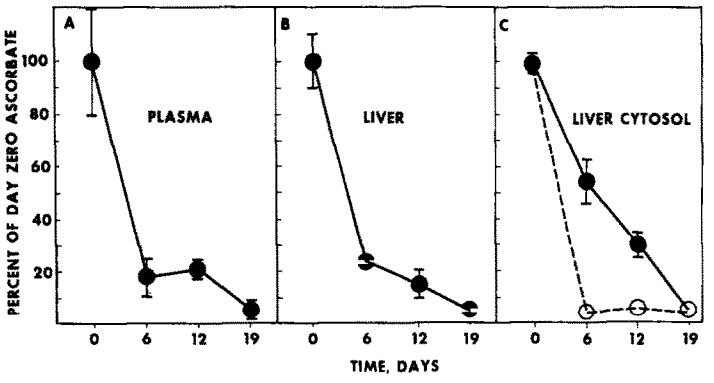

Fig. 1. Effect of AA depletion on plasma, liver and liver cytosol AA in guinea pigs. Dots represent means ± S.E.M. The dashed line represents the difference between total liver AA and liver cytosol AA, expressed as per cent of day zero AA. Initial AA values were: plasma, 6.1 ± 0.34 μg/ml; liver, 314 ± 3.2 μg/g; and liver cytosol, 114.7 ± 3.4 μg/g. Open circles represent the difference in total liver AA and cytosolic AA.

activity in 6, 12 and 19 days respectively (Table 1). Likewise, microsomal cytochrome P-450 content decreased 86, 65 and 47 per cent of normal activity in 6, 12 and 19 days respectively. As shown in Fig. 1, plasma AA seems to parallel liver AA. Both plasma and liver AA dropped precipitously in 6 days and much less so on days 12 and 19. However, Fig. 1C illustrates that liver cytosol, which represents extracellular and intracellular pools, seems to decrease at a slower rate. The decreases in cytosolic AA were related more closely to decreases in aminopyrine demethylase activity and cytochrome P-450 content (Table 1). There was no significant difference between liver weights of guinea pigs killed 6, 12 and 19 days after being placed on the AA-free diet and those of normal guinea pigs.

After 25 days, liver AA dropped significantly to 5.6 per cent in guinea pigs on the AA-free diets compared to liver AA found in guinea pigs dosed with 25 mg AA/100 g for the same number of days (Table 2). No statistically significantly difference was found in liver weights or body weights between the two dietary groups. Regardless of whether livers were perfused or not perfused, cytochrome P-450 content decreased significantly in the deficient group to 48.5 and 55.2 per cent, respectively, of the values found in the supplemented guinea pigs. Perfusing AA-deficient livers and AA-supplemented livers produced about the same percentage of the original cytochrome P-450 content, i.e. 74 and 84 per cent respectively. We found that perfusing livers from normal guinea pigs resulted in a significant decrease of hepatic AA (71 per cent, $P < 0.05$). Normal values of AA of 154 ± 16 μg/g were reduced to 45 ± 9 μg/g of liver.

MHO activity in livers of AA-deficient animals decreased significantly to 44 per cent of that in livers from AA-supplemented animals (Table 2). Table 3 shows that in a separate experiment the changes in hepatic MHO are apparently dose dependent on AA.

DISCUSSION

Several previous studies have demonstrated an influence of AA on hepatic metabolism of drugs in guinea pigs [1–4]; the present findings have confirmed these earlier studies. However, there is still no satisfactory explanation for what the biochemical basis for the participation of AA in drug metabolism might be. A recent study suggested that, at least in the lung, the

Table 2. Effect of AA on hepatic microsomal cytochrome P-450 in perfused and non-perfused livers, and hepatic microsomal heme oxygenase (MHO) activity*

	Liver wt (g)	AA (μg/g)	Cytochrome P-450 (nmoles/mg microsomal protein)	Heme oxygenase (nmoles bilirubin formed/10 min/mg protein)
AA-deficient animals†				
Non-perfused livers	11.56 ± 0.63	15.2 ± 4.0	0.784 ± 0.030	
Perfused livers			0.579 ± 0.034	0.026 ± 0.008
AA-supplemented animals‡				
Non-perfused livers	13.42 ± 0.99	270.0 ± 25.7§	1.421 ± 0.222§	
Perfused livers			1.194 ± 0.025§	0.059 ± 0.010§

* N = seven to eight animals per group except where indicated. Values are expressed as means ± S.E.M. Animals were killed after 25 days on the dietary regimen.

† Plasma level of AA was 0.9 ± 0.6 μg/ml; N = 16.

‡ Plasma level of AA was 6.2 ± 0.8 μg/ml; N = 12.

§ Significantly different from respective AA-deficient animals ($P < 0.05$).

Table 3. Dose–response dependence of hepatic microsomal heme oxygenase on AA*

AA treatment†	Liver AA (μg/g)	Heme oxygenase‡
0	37.8 ± 3.9	0.043 ± 0.011
1	88.6 ± 19.1	0.082 ± 0.009
50	200.8 ± 23.2	0.108 ± 0.010

* Values are expressed as means ± S.E.M. A distribution-free test for ordered alternatives indicated that there was a significant increase in heme oxygenase activity with increasing doses of AA, $P < 0.005$ [18]. N = five to seven animals per group. Animals were on the AA-deficient diet and specific treatments for 25 days, whereupon liver AA and heme oxygenase activity were measured.

† Dose of AA/day, mg/100 g of body weight.

‡ Expressed in nmoles bilirubin formed/10 min/mg of protein.

extracellular pool of AA is maintained at the expense of other pools in AA depletion [19]. Essentially, the cytosols from our study represent the sum of extracellular and intracellular AA pools, which we found to decrease at a slower rate than AA content of the whole liver in general. Ascorbic acid analysis by the dinitrophenylhydrazine (DNPH) method lacked the sensitivity to determine directly the AA content of subcellular fractions in this study. However, the difference between total liver AA and cytosolic AA represents the subcellular component, i.e. the sum of nuclear-cellular debris, microsomes, etc. This calculation, shown as the dotted line in Fig. 1C, suggests that noncytosolic AA drops precipitously, as do the total liver AA levels.

As found in previous studies [4, 20], the decrease in the rate of drug metabolism and the rate of decrease in the content of cytochrome P-450 appear related to AA depletion, particularly by loss of cytosolic AA (Table 1). We also found that perfusion of livers resulted in only 29 per cent of hepatic AA being retained. Perfusion of livers from AA-deficient or AA-supplemented guinea pigs decreased cytochrome P-450 content to 48.5 and 55.52 per cent of the non-perfused values respectively. If we assume that liver perfusion results in the removal of extracellular AA, then the extracellular pool and perhaps the intracellular pool have an important influence on drug metabolism and cytochrome P-450 content. Alternately, the perfusion *per se* may just reduce liver drug metabolism and cytochrome P-450 content by a loss of blood constituents, such as white cells, that have significant enzyme activity, or by a loss due to partial hepatocyte destruction and subsequent washout of intracellular microsomal material. Relevant to our results are the findings reported recently [21], demonstrating that the *in vitro* microsomal hydroxylation of *p*-nitrophenol and the demethylation of ethylmorphine were increased by the addition of AA to the reaction mixture, and the increase was AA concentration dependent. In addition, this study showed that drug metabolism was restored by the addition of AA to liver microsomes previously depleted of AA, and that the addition of the liver cytosol fraction enhanced the activity of microsomes.

The ratio of AA to cytochrome P-450 is about 2, regardless of whether the cytochrome is obtained from AA-deficient or AA-supplemented guinea pigs [22]. Also, there is no change produced by AA deficiency in the kinetics of drug metabolism and essentially no change in drug metabolism cytochrome P-450 binding spectrum [2, 22]. These results suggest that a quantitative change rather than a qualitative change may occur in cytochrome P-450 obtained from AA-deficient animals. Therefore, we examined whether or not AA deficiency might influence the catabolism of cytochrome P-450 [23]. In selenium-deficient rats, it has been found that MHO is increased several-fold and consequently there is reduced cytochrome P-450 content due to increased catabolism of cytochrome heme [24, 25]. We found that heme catabolism was not induced with AA deficiency, but rather that MHO is related directly statistically, to liver AA levels (Table 3). The increase of MHO with increasing liver AA may be related to the increased cytochrome P-450 content, since MHO activity depends on heme load and increasing its activity is thought to indicate stimulated rates of heme catabolism [26]. An alternative explanation is that AA directly influences MHO activity.

Luft *et al.* [27] reported that the administration of a precursor of heme, δ-aminolevulinic acid (ALA), to AA-deficient guinea pigs caused an increase in the quantity of cytochrome P-450 content. They suggested that AA may be important in the formation of precursors for heme synthesis. Recent studies based on that hypothesis have failed to find any impairment of heme synthesis in AA-deficient animals [8, 28]. There was no change in ALA dehydratase, ALA synthetase, or ferrochelatase.

No qualitative changes in cytochrome P-450 and no changes in heme synthesis can explain the decrease of cytochrome P-450 content. In this study, we found no evidence to suggest that increased heme catabolism, at least via induction of MHO activity with subsequently increased cytochrome P-450 heme breakdown, is induced in AA-deficient animals. Alternately, the increased breakdown of cytochrome P-450, by conversion of heme into abnormal derivatives, might be described as "green pigment" [29]. These derivatives have been found in microsomes isolated from livers of rats injected with 2-allyl-2-isopropylacetamide [30]. Investigations have shown that such preparations of cytochrome P-450 are undergoing lipid peroxidation [13]. If lipid peroxidation is the mechanism of green pigment formation, then it is unlikely that AA deficiency results in the increased degradation of cytochrome P-450 via green pigment formation, since others have failed to detect any other evidence of oxidative lipid damage [28]. These combined results indicate that consideration should be directed to possible problems that might occur in the synthesis of the apoprotein for cytochrome P-450 [32].

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